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(54) Title: INTERACTION BETWEEN CYCLIN D1 AND STEROID RECEPTOR CO-ACTIVATORS AND USES THEREOF IN ASSAYS

(57) Abstract

The present invention relates to the finding that cyclin D1 interacts in a ligand–independent fashion with coactivators of the SRC–1 family. The direct interaction of cyclin D1 enhances estrogen receptor (ER) mediated transcription and provides a novel target for the development of assays for substances which modulate the cell cycle. The invention provides assay methods for the prevention of growth of tumours, for assays for compounds useful in the prevention of tumours and compounds obtainable by such assays.

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INTERACTION BETWEEN CYCLIN D1 AND STEROID RECEPTOR CO-ACTIVATORS AND USES THEREOF IN ASSAYS

The present invention relates to methods for preventing the growth of tumours, and for assays for compounds useful in the prevention of tumours.

Background to the invention.

5   The cyclins are a class of polypeptides which are involved in the control of the cell cycle. Three closely related human D-type cyclins have been identified, all of which interact with and activate cyclin dependent kinases (CDK) 4 and 6, although they have specialized function in distinct cell types. The

10 cyclin D1 gene has been found to be overexpressed and/or deregulated by clonal chromosome rearrangements or by amplification in B cell lymphoma, in parathyroid adenoma, and in breast and squamous cell cancer. It has also recently been shown that cyclin D1 deficient mice have a defect in estrogen-

15 mediated proliferation of breast epithelium during pregnancy (Sicinski et al, 1995, Cell 82; 621-630; Fantl et al, 1995, Genes & Development 9; 2364-2372).

Cyclin D1 is induced in response to mitogenic stimulation of quiescent cells and acts as an activator of CDK4 and CDK6.

20 These cyclin D1/CDK complexes are key regulators of progression through the G1 phase of the cell cycle and are involved in functional inactivation of the retinoblastoma family proteins (reviewed in (Beijersbergen and Bernards, 1996)). Cyclin D1 is amplified or over-expressed in a number of human malignancies,

25 the most prominent being breast cancer, in which up to 50% of all cases have elevated levels of cyclin D1 (Buckley et al., 1993; Schuuring et al., 1992; van Diest et al., 1997). The relevance of cyclin D1 over-expression is underscored by the finding that tissue-specific transgenic expression of cyclin D1

30 in mice results in mammary hyperplasia and adenocarcinoma (Wang et al., 1994). Furthermore, *cyclin D1* knockout mice show a marked defect in breast epithelium development during pregnancy and cyclin D1 reduces mitogen requirement of breast cancer cell

lines (Fantl et al., 1995; Musgrove et al., 1994; Sicinski et
al., 1995; Zwijsen et al., 1996). Cyclin D1 is over-expressed
preferentially in ER-positive breast cancers, suggesting that
cyclin D1 derives (part of) its oncogenic activity in breast
5   cancer by acting on ER (Gillett et al., 1996; van Diest et al.,
1997). We and others have recently made a connection between ER
and cyclin D1 by showing that cyclin D1 can interact directly
with the ligand binding domain of ER and can stimulate ER
transactivation in a ligand-independent and CDK-independent
10 fashion (Neuman et al., 1997; Zwijsen et al., 1997).

WO97/40378 also discloses that cyclin D1 interacts with the
estrogen receptor (ER). Transcription is increased by
formation of cyclin D1-ER complex which binds to the estrogen
response element (ERE) found upstream of estrogen responsive
15 genes. This finding provides a target for the control of cell
proliferation, particularly in those cells which grow in
response to stimulation by estrogen, e.g. breast tumour cells.

Several lines of evidence suggest that efficient
transactivation requires additional positively acting factors
20 termed coactivators (Pugh and Tjian, 1990). Several candidate
steroid receptor coactivators (SRCs) have been identified. The
first coactivator identified based on its ability to interact
with the progesterone receptor was SRC-1 (Onate et al., 1995;
Yao et al., 1996). This protein is the founding member of a
25 family of related SRCs that include TIF-2/GRIP-1 (Hong et al.,
1997; Voegel et al., 1996) and AIB-1/ACTR/RAC-3/p/CIP (Anzick
et al., 1997; Chen et al., 1997; Li et al., 1997; Torchia et
al., 1997). Several functional domains are highly conserved in
all members of this family. For instance, the N-terminal
30 regions contain basic helix-loop-helix (bHLH) and Per-ARNT-Sim
(PAS) domains. Both motifs are thought to be involved in
protein-protein interactions and DNA-protein interactions (Yao
et al., 1996). Interestingly, the bHLH-PAS domain is
dispensable for SRC-1 activity, including receptor interaction
35 and receptor activation (Onate et al., 1995; Yao et al., 1996).

In addition, all SRCs contain multiple LXXLL motifs (L is
leucine; X is any amino acid) in the central region of the
protein. These motifs were recently shown to be involved in
nuclear receptor interaction (Heery et al., 1997; Le Douarin et
5   al., 1996; Torchia et al., 1997). Besides ER, SRC-1 also
interacts with another coactivator of steroid receptors,
CBP/p300, and both types of coactivators act synergistically to
enhance ER transactivation (Chakravarti et al., 1996; Chen et
al., 1997; Hanstein et al., 1996; Kamei et al., 1996; Smith et
10 al., 1996; Yao et al., 1996). Both coactivators of the SRC-1
family and the p300/CBP family have intrinsic histone acetyl
transferase (HAT) activity which is widely believed to be
involved in chromatin remodeling during transcriptional
activation (Jenster et al., 1997; Ogryzko et al., 1996; Spencer
15 et al., 1997).

Disclosure of the Invention.
We have continued to investigate the mechanism of ER activation
by cyclin D1 and found that surprisingly, there is also a
direct interaction between SRC-1 and cyclin D1.   This
20 interaction appears to be mediated primarily through the LXXLL
motifs of SRC-1. We have also found evidence that other SRCs,
particlarly AIB-1 and TIF-2 (which also have LXXLL motifs) bind
to cyclin D1 in a similar manner. The direct interaction of
cyclin D1 with SRCs enhances ER mediated transcription. The
25 interaction provides a novel target for the development of
assays for substances which modulate the cell cycle,
particularly in cells which grow in response to stimulation by
estrogen, e.g. breast tumour cells.

Thus the present invention is useful for assaying for potential
30 modulators of the growth of estrogen responsive tumour cells,
particularly those in which cyclin D1 is found at elevated
levels. Elevated levels of cyclin D1 may occur for a variety
of reasons, e.g. over-expression of a single cyclin D1 gene or
by gene amplification.

35 Thus in a first aspect the present invention provides an assay
for a modulator of estrogen responsive tumour cells which

comprises:

a)   bringing into contact a cyclin D1, an SRC and a putative
      modulator compound under conditions where the cyclin D1
      and the SRC, in the absence of modulator, are capable of

5   forming a complex; and

b)   measuring the degree of inhibition of complex formation
      caused by said modulator compound.

The present invention further provides an assay for a modulator
of estrogen responsive tumour cells which comprises:

10   a)   bringing into contact a cyclin D1, an SRC, an estrogen
      receptor and a putative modulator compound under
      conditions where the cyclin, the SRC and the estrogen
      receptor, in the absence of modulator, are capable of
      forming a complex which is capable of binding to an

15   estrogen response element;

b)   providing an estrogen response element to which the
      complex is capable of binding and transcriptionally
      activating; and

c)   measuring the degree of inhibition of binding or

20   transcriptional activation caused by said modulator
      compound.

In a further aspect, the invention provides compounds
obtainable by such an assay, for example peptide compounds
based on the portions of cyclin D1, an SRC, or the estrogen

25   receptor which interact with each other.

The assay of the invention is optionally performed in the
presence of an estrogen which is capable of binding to the
estrogen receptor.

Detailed Description of the Invention.

30   *Cyclin D1*

The cyclin D1 may be any suitable mammalian cyclin D1,
particularly human cyclin D1. Human D1 cyclin has been cloned
and sources of the gene can be readily identified by those of
skill in the art. See for example, Xiong et al, 1991, Cell 65;

691-699 and Xiong et al, 1992, Genomics 13; 575-84. Murine D1
cyclin has also been cloned. Other mammalian cyclins can be
obtained using routine cloning methods analogous to those
described in the aforementioned references.

5   Although wild-type cyclin D1 is preferred mutants and variants
     of D1 which still retain the ability to interact directly with
     the estrogen receptor may also be used. Examples of cyclin D1
     mutants are well known in the art and two particular mutants
     are illustrated in WO97/40378. A particularly preferred mutant
10   is a mutant which carries a mutation in the cyclin box, such as
     the cyclin D1-KE mutant. This mutation renders the D1 unable
     to bind CDKs.

     Mutants and other variants will generally be based on wild-type
     mammalian cyclin D1s and have a degree of amino acid identity
15   which is desirably at least 70%, preferably at least 80%, 90%,
     95% or even 98% homologous to a wild type mammalian cyclin.

     It is not necessary to use the entire cyclin D1 proteins
     (including their mutants and other variants) for assays of the
     invention. Fragments of the cyclin may be used provided such
20   fragments retain the ability to interact with the target domain
     of the SRC and desirably also the estrogen receptor responsible
     for the cyclin interaction. Fragments of cyclin D1 may be
     generated in any suitable way known to those of skill in the
     art. Suitable ways include, but are not limited to,
25   recombinant expression of a fragment of the DNA encoding the
     cyclin. Such fragments may be generated by taking DNA encoding
     the cyclin, identifying suitable restriction enzyme recognition
     sites either side of the portion to be expressed, and cutting
     out said portion from the DNA. The portion may then be
30   operably linked to a suitable promoter in a standard
     commercially available expression system. Another recombinant
     approach is to amplify the relevant portion of the DNA with
     suitable PCR primers. Small fragments of the cyclin (up to
     about 20 or 30 amino acids) may also be generated using peptide
35   synthesis methods which are well known in the art. Generally
     fragments will be at least 40, preferably at least 50, 60, 70,

80 or 100 amino acids in size.

Particularly preferred fragments include those which are based
upon the C-terminal region of cyclin D1, said region including
the C-terminal motif LLXXXL, which in human cyclin D1 is
5   represented by the sequence LLESSL at residues 254-259. These
fragments will be able to interact with the SRC and desirably
also the ER. Desirably the fragments comprise the C-terminal
region of cyclin D1.

The ability of suitable fragments to bind to the SRC (or
10   fragment thereof) may be tested using routine procedures such
as those described in the accompanying examples relating to
intact cyclin D1. Reference herein to cyclin D1 includes the
above mentioned mutants and fragments which are functionally
able to bind the SRC unless the context is explicitly to the
15   contrary.

SRC Protein
The SRC protein may be any human or other mammalian protein, or
fragment thereof which has the ability to bind to a steroid
receptor and cyclin D1, and enhance the receptor's
20   transcriptional activity. A number of SRC proteins have been
cloned. These include SRC-1, the sequence of which is shown in
Onate *et al*, *ibid*, 1995; TIF-2, the sequence of which is shown
in Voegel *et al*, *ibid*, 1996, and AIB-1 (also called ACTR), the
sequence of which is shown in Chen *et al*, *ibid*, 1997. These
25   are all human SRC proteins. The sequence of the murine
homologue of TIF-2 is disclosed in Hong *et al*, *ibid*, 1997
(where it is called GRIP-1), and the sequence of the murine
homologue of AIB-1 is disclosed in Torchia *et al*, *ibid*, 1997
(where it is called p/CIP).

30   There are a number of common structural features found in the
SRC proteins identified to date. These include an N-terminal
region of about 300 amino acids comprising the bHLH and PAS
regions mentioned above. A comparision of these domains is
shown in Chen *et al*, *ibid*. The AIB-1 bHLH/PAS domain shares

about 65% homology (identity) with the corresponding TIF-2
domain and about 58% homology (identity) with the corresponding
SRC-1 domain.

5   The central portion of the proteins (from about amino acids 570
     to 780 of SRC-1) comprises a receptor interaction domain (RID)
     which also appears to be primarily responsible for the physical
     interaction with cyclin D1. The AIB-1 RID has 42% and 27%
     amino acid identity with the TIF-2 and SRC-1 RIDs respectively.
     The RIDs comprise three motifs which share the common primary
10   sequece LXXLL.

     The SRCs also have, C-terminal to the central portion, a
     histone acetyl transferase domain.

     Thus an SRC protein in its broadest sense is one which has, in
     N- to C- terminal order, a bHLH/PAS domain of about 300 amino
15   acids with at least 50% homology to the corresponding domain of
     SRC-1, AIB-1 or TIF-2; a centrally located RID of about 210
     amino acids with one or more, such as three, LXXLL motifs; and
     a domain which has HAT activity.

     Variants of the above SRCs may be used, such as synthetic
20   variants which have at least 50% amino acid identity to a
     naturally occurring SRC (particularly a human SRC), preferably
     at least 60%, 70%, 80%, 90%, 95% or 98% identity. The assay
     preferably uses the same mammalian source SRC as the cyclin D1.
     A preferred subset of variants are those which retain one or
25   more of the motifs LXXLL of the central portion, and preferably
     retains three as found in the SRCs mentioned above. The
     variants may also be manipulated to provide more than three,
     for example, four, five or six, LXXLL motifs. These additional
     motifs will preferably be within the RID domain, but may also
30   be introduced in other portions of an SRC protein. The motifs
     may be introduced by insertion of additional amino acids of by
     substitution of amino acids within the SRC.

     Fragments of the SRC protein and its variants may be used,
     provided that said fragments retain the ability to interact

with a wild-type cyclin D1, preferably wild-type human cyclin
D1. Such fragments are desirably at least 50, preferably at
least 75, 100, 200, 250 or 400 amino acids in size. Desirably
such fragments include one or more of the (such as all three of
5 the centrally located) LXXLL motifs.

Estrogen Receptor

The estrogen receptor used in the assay may be obtained from
the same mammalian source as the cyclin D1. The human estrogen
receptor is preferred. This is a 66 kd protein which functions
10 as hormone-activated transcription factor, the sequence of
which is published in the art and is generally available.
Receptor activation is thought to be a consequence of ligand-
induced conformational changes in the structure of the
receptor. The complex of estrogen with its receptor binds to
15 specific DNA sequences including, with high affinity, to a
well-defined 13-bp palindromic sequence - the estrogen response
element (ERE). The ERE is usually located upstream of an
estrogen-responsive gene. Estrogen responsive genes include
progesterone receptor and PS-2. Transcriptional activation of
20 these genes is involved in estrogen-responsive tumour growth.

There is also a second estrogen receptor, ER beta. This is
disclosed in Kuiper *et al*, 1996. The ERβ may also be used, and
is included herein when reference is made to the ER.

Fragments and variants of the estrogen receptor which retain
25 the ability to interact with the cyclin D1 may also be used,
and such fragments and mutants may be obtained by methods
analogous to those described above in relation to cyclin
fragments.

Variants of ER include a number of variants which have been
30 found to be associated with breast cancer. Some of these are
associated with breast cancers which are resistant to anti-
estrogen therapies, particularly tamoxifen therapies. Variants
are disclosed in, for example, McGuire *et al*, 1991; Fuqua *et
al*, 1993; and Miksicek *et al*, 1995.

Mutants and other variants of ER will generally be based on wild-type mammalian ERs and have a degree of amino acid identity which is desirably at least 70%, preferably at least 80%, 90%, 95% or even 98% homologous to a wild type mammalian
5   ER.

Particluarly preferred fragments (including fragments of the above-described variants) include those which retain the E/F regions comprising amino acids 292-595 or a portion thereof such as 340-595 of the ER. Smaller fragments may also be used,
10   e.g. those starting at around 292, 300, 340, 360 or 380 and ending at the C-terminus (595) or a truncation thereof, e.g. 590, 580, 550 or 500. Suitable fragments may be determined by routine experimentation. Reference herein to the estrogen receptor includes fragments and mutants which retain the
15   ability to interact with cyclin D1 unless the context is explicitly to the contrary. In this context, "interact" includes binding to ER, the minimum requirement for an *in vitro* assay. For *in vitro* or *in vivo* assays which rely on the D1-SRC-ER complex binding to an ERE and activating transcription,
20   the necessary interaction must provide this.

In an alternative format the ER may be added in the form of two partial proteins, for example expressed from two partial ER expression vectors. The first protein encodes the N-terminal region of ER (containing AF-1 and the DNA binding region), the
25   second the C-terminal part (containing AF-2 and the hormone binding region). The hormone binding region is optionally linked to an activator protein such as the GAL4 activation domain or the VP16 activation domain. As illustrated in the accompanying examples, ERE-dependent transcription is
30   stimulated by both cyclin D1 and an SRC protein, and the presence of both components synergistically enhances the activation. Thus putative modulators may be used and the decrease in the synergistic enhancement of activation may be observed in assays of the present invention.

35   *Estrogens*

Although we previously have found that the estrogen receptor is activated by cyclin D1 alone to provide ERE-responsive gene transcription we have found that transcription is enhanced synergistically in the presence of an estrogen. It is thus a

5   preferred aspect of the assay that estrogen is also brought into contact with the cyclin D1, SRC, estrogen receptor and putative inhibitor compound when a transcriptional acitivation assay of the invention is performed. The estrogen may be any natural or synthetic estrogen capable of binding to and

10 activating the estrogen receptor. Examples of estrogens include 17β-estradiol.

Assay formats

One assay format which is widely used in the art to study the interaction of two proteins is a two-hybrid assay. This assay

15 may be adapted for use in the present invention. A two-hybrid assay comprises the expression in a host cell of the the two proteins, one being a fusion protein comprising a DNA binding domain (DBD), such as the yeast GAL4 binding domain, and the other being a fusion protein comprising an activation domain,

20 such as that from GAL4 or VP16. In such a case the host cell (which again may be bacterial, yeast, insect or mammalian, particularly yeast or mammalian) will carry a reporter gene construct with a promoter comprising a DNA binding elements compatible with the DBD. The reporter gene may be a reporter

25 gene such as chloramphenical acetyl transferase, luciferase, green fluorescent protein (GFP) and β-galactosidase, with luciferase being particularly preferred.

Two-hybrid assays may be in accordance with those disclosed by Fields and Song, 1989, Nature 340; 245-246. In such an assay

30 the DNA binding domain (DBD) and the transcriptional activation domain (TAD) of the yeast GAL4 transcription factor are fused to the first and second molecules respectively whose interaction is to be investigated. A functional GAL4 transcription factor is restored only when two molecules of

35 interest interact. Thus, interaction of the molecules may be measured by the use of a reporter gene operably linked to a GAL4 DNA binding site which is capable of activating

transcription of said reporter gene.

Thus two hybrid assays may be performed in the presence of a
potential modulator compound and the effect of the modulator
will be reflected in the change in transcription level of the
5   reporter gene construct compared to the transcription level in
the absence of a modulator.

Host cells in which the two-hybrid assay may be conducted
include mammalian, insect and yeast cells, with yeast cells
(such as *S.cerivissiae* and *S.pombe*) being particularly
10   preferred.

In the case of the present invention, a two-hybrid assay will
be conducted by fusing the cyclin D1 protein being used to a
DNA-binding domain and the SRC protein to the activation
domain.

15   Another assay format measures directly, *in vivo* or *in vitro* the
interaction between cyclin D1 and the SRC by labelling one of
these proteins with a detectable label and bringing it into
contact with the other protein which has been optionally
immobilised on a solid support, either prior to or after
20   proteins have been brought into contact with each other.
Suitable detectable labels include ^{35}S-methionine which may be
incorporated into recombinantly produced proteins, and tags
such as an HA tag, GST or histidine. The recombinantly
produced protein may also be expressed as a fusion protein
25   containing an epitope which can be labelled with an antibody.
Alternatively, an antibody against the cyclin and/or SRC can be
obtained using conventional methodology.

The protein which is optionally immobilized on a solid support
may be immobilized using an antibody against that protein bound
30   to a solid support or via other technologies which are known
per se. In the Examples which follow a preferred *in vitro*
interaction is illustrated which utilises a fusion protein of
SRC-1 fused to glutathione-S-transferase (GST). This may be

immobilized on glutathione agarose beads. In an *in vitro* assay
format of the type described above the putative inhibitor
compound can be assayed by determining its ability to diminish
the amount of labelled cyclin D1 which binds to the immobilized
5   GST-SRC-1. This may be determined by fractionating the
glutathione-agarose beads by SDS-polyacrylamide gel
electrophoresis. Alternatively, the beads may be rinsed to
remove unbound protein and the amount of protein which has
bound can be determined by counting the amount of label present
10   in, for example, a suitable scintillation counter.

Another assay format is dissociation enhanced lanthanide
fluorescent immunoassay (DELFIA) (Ogata et al, 1992). This is
a solid phase based system for measuring the interaction of two
macromolecules. Typically one molecule (either SRC1 or cyclin
15   D1) is immobilised to the surface of a multi well plate and the
other molecule is added in solution to this. Detection of the
bound partner is achieved by using a label consisting of a
chelate of a rare earth metal. This label can be directly
attached to the interacting molecule or may be introduced to
20   the complex via an antibody to the molecule or to the molecules
epitope tag. Alternatively, the molecule may be attached to
biotin and a streptavidin-rare earth chelate used as the label.
The rare earth used in the label may be europium, samarium,
terbium or dysprosium. After washing to remove unbound label,
25   a detergent containing low pH buffer is added to dissociate the
rare earth metal from the chelate. The highly fluorescent
metal ions are then quantitated by time resolved fluorimetry.
A number of labelled reagents are commercially available for
this technique, including streptavidin, antibodies against
30   glutathione-S-transferase and against hexahistidine.

In an alternative mode, the one of the cyclin D1 and the SRC
may be labelled with a fluorescent donor moiety and the other
labelled with an acceptor which is capable of reducing the
emission from the donor. This allows an assay according to the
35   invention to be conducted by fluorescence resonance energy
transfer (FRET). In this mode, the fluorescence signal of the
donor will be altered when the cyclin D1 and an SRC interact.

The presence of a candidate modulator compound which modulates
the interaction will increase or decrease the amount of
unaltered fluorescence signal of the donor.

FRET is a technique known per se in the art and thus the
5   precise donor and acceptor molecules and the means by which
they are linked to the cyclin D1 and an SRC may be accomplished
by reference to the literature.

Suitable fluorescent donor moieties are those capable of
transferring fluorogenic energy to another fluorogenic molecule
10 or part of a compound and include, but are not limited to,
coumarins and related dyes such as fluoresceins, rhodols and
rhodamines, resorufins, cyanine dyes, bimanes, acridines,
isoindoles, dansyl dyes, aminophthalic hydrazines such as
luminol and isoluminol derivatives, aminophthalimides,
15 aminonaphthalimides, aminobenzofurans, aminoquinolines,
dicyanohydroquinones, and europium and terbium complexes and
related compounds.

Suitable acceptors include, but are not limited to, coumarins
and related fluorophores, xanthenes such as fluoresceins,
20 rhodols and rhodamines, resorufins, cyanines,
difluoroboradiazaindacenes, and phthalocyanines.

A preferred donor is fluorescein and preferred acceptors
include rhodamine and carbocyanine. The isothiocyanate
derivatives of these fluorescein and rhodamine, available from
25 Aldrich Chemical Company Ltd, Gillingham, Dorset, UK, may be
used to label the cyclin D1 and ER. For attachment of
carbocyanine, see for example Guo et al, J. Biol. Chem., 270;
27562-8, 1995.

The assays described above may also utilise the requirement of
30 SRC and ER for an estrogen if binding of ER to the estrogen
response element (ERE) is to occur. We have found that the
binding of ER to the ERE is independent of estrogen when either
cyclin D1 is present, or both cyclin D1 and an SRC are present.
The binding of ER to the ERE provides the basis for a binding

assay. For example, a detectably labelled ERE may be brought into contact with ER, cyclin D1 and an SRC under conditions where, in the absence of an inhibitor, a complex of the three components is detectable on the ERE. The ERE is preferably

5   labelled with a label which allows physical recovery of the ERE, for example with a paramagnetic bead or a protein which may be reversibly or irreversibly attached to a solid support such as a chromatography column.

Once the ERE is recovered, the proteins still associated with

10   it may be recovered and the presence or absence of specific components of the complex determined. Protein components may conveniently be examined by Western blotting. Alternatively, immunological means may be used. In another alternative, the different protein components may be labelled with different

15   fluorogenic or radioactive label, which may be detected with little or no separation of the recovered ERE-protein complex.

Such assays may be used to determine whether, in the presence of a putative modulator compound, the SRC remains bound to cyclin D1 in the ternary complex.

20   The assay of the invention may also take the form of an *in vivo* assay based on the ability of the complex of the ER, cyclin D1 and the SRC to activate estrogen responsive transcription. The term "*in vivo*" includes cell lines and the like, and excludes whole humans. The *in vivo* assay may be performed in an

25   estrogen responsive cell line which expresses the estrogen receptor or in ER-negative cell lines in which the ER is expressed from a vector introduced into the cell. The cell line may be in tissue culture or may be a cell line xenograft in a non-human animal subject.

30   Where a cell line expressing the estrogen receptor is used a reporter gene construct comprising an ERE operably linked to a reporter gene may be introduced into the cell together with vector(s) for the expression of a cyclin D1 and an SRC. These proteins may be expressed from a single vector or from two

separate vectors. The vector(s) may utilize any suitable
promoter, such as described herein. Two or more EREs (for
example 3, 4 or 5) may be present in the receptor construct and
this may enhance sensitivity of the assay. The reporter gene
may be any suitable reporter gene used in the art. Such
reporter genes includes chloramphenicol acetyl transferase
(CAT), β-galactosidase, luciferase or GFP. The cyclin D1
expression vector will comprise DNA encoding cyclin operably
linked to a promoter capable of expressing the gene in the host
cell. Suitable promoters include viral promoters such as a CMV
or SV40 promoter.

The cell lines used in assays of the invention may be used to
achieve transient expression of the cyclin although in a
further aspect of the invention cells which are stably
transfected with constructs which express a D1 and SRC and,
where required, the ER may also be generated. Means to
generated stably transformed cell lines are well known in the
art and such means may be used here.

Suitable cell lines include breast cancer cell lines which are
widely available in the art. The Examples which follow utilise
the T47D breast cancer cell line although other suitable
Examples include MCF-7 and MDA. Such assays may also be
performed in cell free systems such as a reticulocyte cell free
system.

Where the cell line does not express ER, a construct capable of
expressing this protein may also be introduced into the cell
operably linked to a suitable promoter.

The precise format of the assays of the invention may be varied
by those of skill in the art using routine skill and knowledge.
In the *in vitro* assays of the invention, the amount of cyclin
D1, SRC and, where required, estrogen receptor may be varied
depending upon the scale of the assay. In general, the person
of skill in the art will select relatively equimolar amounts of
the two components, say from 1:10 to 100:1, preferably from 1:1
to 10:1, molar ratio of cyclin D1 to SRC. However there may be

particular assay formats which can be practiced outside this
range.

In the *in vivo* assays of the invention, it will be desirable to
achieve sufficient expression of cyclin D1 to recruit
5   sufficient SRC to a complex with ER that the effect of a
putative modulator compound may be measured. Where the cell
does not express ER, sufficient expression of this will also be
required. The level of expression of cyclin D1 and SRC (and
where necessary ER) may be varied within fairly wide limits, so
10   that the intracellular levels of the two may vary by a wide
ratio, for example from 1:10 to 1000:1, preferably 1:1 to
100:1, molar ratio of cyclin D1 to SRC.

While not wishing to be bound by any one theory, the
interaction of SRC to both ER and cyclin D1 through similar
15   regions and motifs of SRC suggests that SRC interaction with
these components may occur through dimerization of SRC. It may
therefore be desirable when performing the invention,
particularly in the presence an ER, to ensure sufficient excess
of SRC to allow for dimerization to occur. In addition, the
20   dimerization of SRC gives rise to a further novel target for
potential modulator compouds, wherein such assays may be
conducted as described herein for SRC-cyclin D1 assays, with
the D1 component being replace by a second SRC, either
identical to or different from the first SRC.

25   The amount of putative modulator compound which may be added to
an assay of the invention will normally be determined by trial
and error depending upon the type of compound used. Typically,
from about 0.01 to 100 nM concentrations of putative modulator
compound may be used, for example from 0.1 to 10 nM. Modulator
30   compounds may be those which either agonise or antagonise the
interaction. Antagonists (inhibitors) of the interaction are
particularly desirable.

Modulator compounds which may be used may be natural or
35   synthetic chemical compounds used in drug screening programmes.
Extracts of plants which contain several characterised or

uncharacterised components may also be used. Modulators which
are putative inhibitor compounds can be derived from the cyclin
D1 and SRC protein sequences. Peptide fragments of from 5 to
40 amino acids, for example from 6 to 10 amino acids from the
5   region of cyclin D1 and SRC which are responsible for the
interaction between these proteins may be tested for their
ability to disrupt this interaction. Antibodies directed to
the site of interaction in either protein form a further class
of putative inhibitor compounds. Candidate inhibitor
10 antibodies may be characterised and their binding regions
determined to provide single chain antibodies and fragments
thereof which are responsible for disrupting the interaction
between cyclin D1 and SRC.

A particular class of peptide compounds will be those based
15 upon the "LXXLL" peptides shown in the accompanying examples.
Such peptides are preferably from 5 to 20 amino acids in size,
and are of the structure Z^1-LXXLL-Z^2 where Z^1 is an N-terminal
of a sequence of from 1 to 8 amino acids, preferably a sequence
found immdiately N-terminal to a naturally occurring LXXLL
20 motif in an SRC, and Z^2 is a C-terminal or sequence of from 1
to 8 amino acids, preferably a sequence found immediately C-
terminal to a naturally occuring LXXLL motif in an SRC or other
nuclear receptor interacting protein such as p300 or CBP. Z^1
and Z^2 may be from the same or different SRC and where from the
25 same, from the same or different motif.

Particular peptides according to this aspect of the invention
include those shown in Table 1 below (the peptides are shown in
conventional N- to C- terminal order using the standard 1-
letter code:

30 | Table 1 |
    | --- |

A A S K H K Q L S E L L R S G	SEQ ID NO:1
S H K L V Q L L T T T A E Q	SEQ ID NO:2
E R H K I L H R L L Q E G S	SEQ ID NO:3
K D H Q L L R Y L L D K D E	SEQ ID NO:4
P Q A Q Q K S L Q Q L L T	SEQ ID NO:5

In a preferred aspect of the invention, Z^1 comprises from 3 to
7 amino acids and contains at least one, such as 1, 2 or 3,
residue selected from the group of K and H. In this embodiment
of the invention, it is preferred that the amino acid at
position -1, -2 or -3, preferably -2, with respect to the first
L of the LXXLL motif is K or H.

Although the second and third amino acids of the LXXLL motif
may vary, it is preferred that at least one of said amino acids
is charged.

We have also found that peptides of this aspect of the
invention are active even where Z^2 comprises a single amino
acid. Thus, in a preferred aspect of the invention, Z^2 may be
from 1 to 4 amino acids in length.

The abovementioned preferred features may be present separately
or in any combination in peptides of the invention.

Peptides comprising an LXXLL motif may comprise multimers of
this motif, either in the form of one or more direct repeats,
of separated by one or more amino acids. The peptides may also
be branched peptide structures, referred to in the art as
dendrimers.

Peptide antagonists of the interaction of cyclin D1 with an SRC
may be linked, at the C- or N-terminal, to a member of the
class of sequences which are membrane translocation sequences
capable of directing a polypeptide through the membrane of a
eukaryotic cell. Example of such polypeptides include the HSV-
1 VP22 protein (Elliot et al, 1997), the HIV Tat protein (for
example residues 1-72 or 37-72, Fawell et al, 1994) or a

sequence that is derived from the *Drosophila melanogaster*
antennapedia protein. The latter is a peptide containing 16
amino acid residues taken from the third helix of the
antennapedia homeodomain protein which translocates across
5   biological membranes (Derossi *et al*, 1994). This translocation
peptide has the sequence:
Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-
Lys. The peptide is preferably joined to the N-terminus of
polypeptides of the invention which antagonize the interaction
10 of cyclin D1 with an SRC.

Other candidate inhibitor compounds may be based on modelling
the 3-dimensional structure of cyclin D1 and SRC and using
rational drug design to provide potential inhibitor compounds
with particular molecular shape, size and charge
15 characteristics.

Candidate modulator compounds obtained according to the method
of the invention may be prepared as a pharmaceutical
preparation. Such preparations will comprise the compound
together with suitable carriers, diluents and excipients. Such
20 formulations form a further aspect of the present invention.
Formulations of inhibitor compounds in particular may be used
in methods of treatment of proliferative diseases, particularly
those involving cells which grow in response to stimulation by
estrogen, for example breast cancer.

25 Candidate inhibitor compounds may also be used in combination
with any other anti-proliferative compounds used to treat
hyperproliferative diseases such as cancers. In such a case,
the assay of the invention, when conducted *in vivo*, need not
measure the degree of inhibition of binding or transcriptional
30 activation caused by the inhibitor compound being tested.
Instead the effect on cell growth or proliferation may be
measured. It may be that such a modified assay is run in
parallel or subsequent to the main assay of the invention in
order to confirm that any effect on cell growth or
35 proliferation is as a result of the inhibition of binding or

transcriptional activation caused by said inhibitor compound and not merely a general toxic effect.

A preferred class of other anti-proliferative compounds are anti-estrogens such as tamoxifens, eg. 4-hydroxytamoxifen or
5   pure anti-estrogens such as N-(n-butyl)-11-[3,17beta-dihydroxy-estra-1,3,5(10)-trien-7alpha-yl]N-methylundecanamide, known as ICI 164,384. Such anti-estrogen compounds may be included in the assay in order to determine inhibitor compounds which may act additively or synergistically with anti-estrogens. This
10   will facilitate the provision of combination therapy against estrogen-responsive tumours.

Estrogen responsive tumours are primarily breast tumours although other tumour types which have been found to be estrogen-responsive include endometrial cancer and ovarian
15   carcinoma.

The interaction between cyclin D1 and the SRC may also be used to monitor the status and progress of disease states associated with enhanced transcription of ERE regulated genes. For example, antibodies may be generated which bind to the
20   cyclin/SRC complex or to one or other component in the allosteric form induced by binding to the other. Such antibodies may be raised in routine ways, using as immunogen the cyclin/SRC complex which may be stabilized by using protein cross-linking reagents. The antibodies generated by such an
25   immunogen may be screened against the immunogen and separately against the cyclin and SRC, in order to obtain antibodies which recognise only the proteins when complexed. Such antibodies may be packaged in kits with other suitable reagents for immunodiagnosis and used in the clinic to monitor disease
30   states.

The following Examples illustrate the invention.

Legends to Figures.
Figure 1. Mapping of the region of cyclin D1 required for ER-mediated transactivation.

(A) The effect of cyclin D1 deletion mutants on ER activation
in the presence of ligand. The cyclin D1 derivatives used in
this study are shown in the left panel; right side of Figure
represents the relative capacity of the mutants to potentiate
5   ERE-dependent transcription. ER-negative COS-7 cells were
transfected with expression vectors for wild type ER (200 ng),
cyclin D1 or cyclin D1 mutants (2.5 μg), an internal control
§-galactosidase plasmid (0.5 μg) and an ERE-TATA-luciferase
reporter (3 μg). The effect on ER transactivation of wild type
10   cyclin D1 was set to 100%. These studies were performed in
three separate experiments and expressed as mean values with
standard deviation less then 10%. The alignment of leucine-rich
motif of ER with D-type cyclins are shown on the bottom; the
leucine-rich motif in cyclin D1 is indicated as a dark box.
15   (B) ER activation by D-type cyclins, cyclin D1 L254/255A point
mutant (D1-LALA) and SRC-1 in the presence of ligand. COS-7
cells were transfected with D-type cyclin expression vectors,
cyclin D1 Leucine-to-Alanine point mutant (D1-LALA) or SRC-1
expression vector together with wild type ER expression vector,
20   an ERE-TATA-luciferase reporter plasmid and the internal
control β-galactosidase construct. Data are expressed as
relative luciferase activity compared with basal ERE-luciferase
activity of wild type ER and are normalized for transfection
efficiencies.

25   Figure 2. Effect of cyclin D1 on helix 12 mutants of ER.
The effect of cyclin D1 on ER mutants was tested in COS-7 cells
(A, B) and in U2-OS cells (C, D) in the presence of ligand. An
ERE-TATA-luciferase reporter construct was used in transient
transfections together with cyclin D1 and ER 1-535 (A, C) or ER
30   L543/544A mutants (B, D). β-Galactosidase served as an internal
control. The reporter activity was determined both in the
presence (black bars) and in the absence (white bars) of 10 nM
17β-estradiol. The relative luciferase activity was calculated
by normalizing to the b-galactosidase activity. The relative
35   reporter activity of wild type ER in the presence of ligand was
used as a reference and set at 100%. In the absence of
transfected ER plasmid, cyclin D1 did not induce
transcriptional activity of the reporter (data not shown). The

transfection were performed in at least five separate
experiments and expressed as average ±SD.

Figure 3. Role of coactivators in cyclin D1-induced
transactivation.
5 (A) Effect of a dominant negative form of SRC-1 (SRC1-DN) on ER
transactivation. SRC1-DN encoding amino acids 1245-1441 of
SRC-1 (1, 2.5 and 5 μg) was introduced by transient
transfection together with wild type ER (200 ng) and was tested
for its ability to modulate ERE-dependent transcription.
10 (B) Effect of SRC1-DN on SRC-1 and TIF 2 mediated ER
transactivation. SRC-1 (3 μg) or TIF-2 (3 μg) were transfected
with SRC1-DN (3 μg) and tested for ER transactivation.
(C) Effect of SRC1-DN on cyclin D1-induced transactivation of
ER 1-535 mutant. Cyclin D1 was cotransfected with SRC1-DN (1,
15 2.5, 5 μg) and tested for its effect on an ER harboring a
deletion of coactivator binding site in helix 12 (ER 1-535).
(D) Effect of SRC1-DN on cyclin D1-induced transactivation of
ER L543/544A mutant. Cyclin D1 and SRC1-DN (1, 2.5, 5 μg) were
transfected and tested for the activity of ER helix 12 point
20 mutant (ER L543/544A). The transient transfections (A-C) were
performed in COS-7 cells, which were maintained in DMEM with 10
nM ligand. The relative activity was calculated by normalizing
to the internal control and was divided by luciferase activity
of ER (mutant) in the presence of ligand. The transfection for
25 each set of conditions were done at least in four independent
experiments and expressed as average ±SD.

Figure 4A.   Structure of GST-SRC1 derivatives used to show that
amino acids 568 to 782 of SRC-1 are required for cyclin D1
binding.

30 Figure 4B. Sequences of peptides P1, P2, P3, P4 and P4M.

Figure 5. Cyclin D1 mediates ligand-independent recruitment of
SRC-1 to ER.
(A) Ligand-independent in vitro binding of SRC-1, ER and cyclin
D1. The purified proteins GST-SRC1, His-tagged cyclin D1 and
35 baculovirus-produced ER were tested for in vitro binding in a

GST-pull down assay. GST protein served as negative control.
Cyclin D1 and ER were incubated with GST-SRC1 in the presence
or absence of 1 μM 17β-estradiol and binding was detected by
Western blot analysis using anti-cyclin D1 and anti-ER
5   monoclonal antibodies. Lane 1 represents 10% of the input for
cyclin D1 and 20% of input of ER proteins.
(B) Cyclin D1 and SRC-1 can interact with DNA-bound ER.
Oligonucleotide containing ER binding sequence was biotin
5'-end labeled and bound to paramagnetic particles coated with
10   streptavidin. Purified GST-SRC1, baculovirus produced ER and
His-tagged cyclin D1 proteins were tested for DNA binding
using these ERE-containing beads and analyzed by Western
blotting using antibodies directed against GST, ER and cyclin
D1, respectively.

15   Figure 6. Role of cyclin D1 in ER transactivation in breast
cancer cells.
The ER-containing T47D and MCF-7 breast cancer cells were
maintained in 17β-estradiol enriched medium with 10% fetal calf
serum after cotransfection with a cyclin D1 L254/255A mutant
20   expression vector and an ERE-reporter gene for testing its
effect on ER activation (left panel). ER transactivation in the
absence of co-expression of the cyclin D1 mutant was set at
100%. The right panel shows the expression levels of endogenous
cyclin D1 in both breast tumor cell lines.

25   Figure 7. Model for cyclin D1-mediated ER transactivation.
In the absence of ligand, ER is unable to interact with SRCs
directly as its leucine-rich coactivator interaction motif is
sterically unavailable for interaction. Ligand-independent
binding of cyclin D1 to ER provides a single leucine-rich
30   interaction motif for SRCs which is present in the carboxyl
terminus of cyclin D1. This results in partial activation of ER
(left panel). Subsequent ligand binding of ER induces a
conformational change in ER that also exposes the leucine-rich
motif in AF-2 of ER for SRC interaction, allowing higher
35   affinity binding of SRCs to the liganded D1/ER complex (right
panel). The observed synergism between estradiol and cyclin D1
in ER activation results from their cooperative recruitment of

SRCs to the D1/ER complex. The protein interaction motifs are shown in italics.

The invention is illustrated by the following examples.

The estrogen receptor (ER) belongs to the steroid/nuclear
5 receptor family of ligand-regulated transcription factors.
Members of this superfamily display a modular structure with
six distinct functional regions (termed A-F), which includes
domains for DNA binding, ligand binding and transcriptional
activation. Like other members of the nuclear hormone receptor
10 superfamily, ER harbors two transcriptional activation
functions (AFs) that act synergistically in transactivation
(Kumar et al., 1987; Tzukerman et al., 1994). Transcriptional
activation is mediated by means of the autonomous activation
function (AF-1) in the N-terminal A/B domain and the ligand-
15 dependent activation function (AF-2) in the C-terminal hormone
binding domain (Evans, 1988; Kumar and Chambon, 1988; Beato,
1989). These two regions flank the DNA-binding domain of the
receptor. Upon ligand binding, ER binds to estrogen responsive
elements, which results in activation of specific ER target
20 genes (Beato, 1989).

It is generally thought that nuclear receptors stimulate
transcription through direct binding to several of the basal
transcription factors, thereby enhancing the formation of a
stable transcription pre-initiation complex (Mitchell and
25 Tjian, 1989). This notion is supported by *in vitro* protein
binding studies which demonstrated that several steroid
receptors interact directly with components of the basal
transcriptional apparatus, including the TATA-box-binding
protein TBP (Sadovsky et al., 1995), TFIIB (Ing et al., 1992;
30 Baniahmad et al., 1993) and human TAF$_{II}$30 (Jacq et al., 1994).
However, as described above, several lines of evidence suggest
that efficient transactivation requires additional positively
acting factors termed coactivators.

Transactivation by steroid/nuclear receptors involves the well-
35 conserved AF-2 domain located in helix 12 of the carboxyl-

terminus of the receptors. It has been demonstrated that helix
12 harbors a leucine-rich motif that constitutes a ligand-
regulated binding site for coactivators, like SRC-1 (Danielian
et al., 1992; Le Douarin et al., 1995; Voegel et al., 1996; vom
5   Baur et al., 1996). Consequently, transactivation by nuclear
receptors is dramatically reduced in receptors that contain
mutations in helix 12 (Danielian et al., 1992; White et al.,
1997). The leucine-rich motif in helix 12 of nuclear receptors
is involved in binding to the LXXLL motifs of the steroid
10   receptor coactivators (Le Douarin et al., 1996; Heery et al.,
1997).

Recently it was found that, apart from cyclin D1, the steroid
receptor coactivator AIB-1 is also frequently amplified in
breast cancer (Anzick et al., 1997). In the present work we
15   describe an unexpected relationship between cyclin D1 and
steroid receptor coactivators which places cyclin D1 at the
center of a complex transcription regulatory network of nuclear
hormone receptors and their coactivators. We identify a novel
functional domain in cyclin D1 that mediates direct interaction
20   with several of the steroid receptor coactivators.

ER and cyclin D1 share a coactivator binding motif.
To study how cyclin D1 activates ER, cyclin D1 deletion mutants
were tested for their effect on ER-transactivation. COS-7 cells
were transfected with cyclin D1 mutants, together with ER and a
25   luciferase reporter gene construct driven by a minimal TATA
promoter linked to an estrogen response element (ERE). Fig. 1A
shows that an amino-terminal deletion mutant of cyclin D1 (D1:
amino acids 91-295) was still able to activate ER, whereas two
carboxyl-terminal deletion mutants of cyclin D1 (D1: amino
30   acids 1-202 and D1: amino acids 1-247) lack ER transactivation
capacity. A cyclin D1 mutant lacking the extreme
carboxyl-terminus (D1: amino acids 1-267) partially retained ER
activation. Together, these data indicate that the domain
required for ER activation is located in the carboxyl-terminal
35   48 amino acids of cyclin D1. This part of the protein is not
involved in CDK interaction and is poorly conserved among the
different cyclins. Alignment of sequences in this part of

cyclin D1 with ER revealed that a motif that resembles the
highly conserved leucine-rich coactivator binding motif in AF-2
of ER is present within the domain of cyclin D1 implicated in
ER transactivation at the amino acid positions 254-259 (Fig.

5   1A). This motif is only partially conserved in cyclin D2 and
D3, two cyclins that are far less active in ER transactivation
(Neuman et al., 1997; Zwijsen et al., 1997). To test the
relevance of this leucine-rich domain of cyclin D1 in ER
activation, a cyclin D1 mutant was constructed in which

10   leucines 254 and 255 were mutated to alanines (D1 L254/255A).
This mutation in cyclin D1 is similar to the mutation in ER (ER
L543/544A) that interferes with coactivator binding to AF-2
(Danielian et al., 1992). In contrast to wild type cyclin D1,
the L254/255A mutant cyclin D1 was virtually unable to activate

15   wild type ER even though this mutant was equally well expressed
and was fully active in other assays (Fig. 1B and see below).
Cyclins D2 and D3, which lack this leucine-rich motif, behaved
similar to the D1 L254/255A mutant in ER activation (Fig. 1B).
These data suggest that cyclin D1 can activate ER through an

20   AF-2-like motif.

Activation of AF-2 mutant ERs by cyclin D1.

It has been demonstrated that AF-2 mutant ERs are unable to
activate transcription, because they cannot recruit steroid
receptor coactivators (SRCs) efficiently (Danielian et al.,

25   1992; vom Baur et al., 1996). To determine the role of the AF-2
domain of ER in cyclin D1-mediated transactivation, a deletion
mutant and a point mutant in the ligand-regulated
carboxyl-terminal activation domain (AF-2) of ER were tested in
COS-7 cells. Fig. 2 shows that, as previously reported, the

30   activity of these AF-2 mutant ERs reflects background levels
(Danielian et al., 1992; vom Baur et al., 1996). Surprisingly,
co-expression of cyclin D1 resulted in a significant activation
of the ER AF-2 deletion mutant (ER 1-535) to levels that were
up to 40% of ligand-activated wild type receptor (Fig. 2A).

35   Cyclin D1 was also able to induce transcription in the absence
of ligand, although this increase was less pronounced.
Similarly, ER L543/544A, which harbors a mutation in the
leucine-rich coactivator binding site in AF-2 (LLXXXL to

AAXXXL), could be activated by cyclin D1 (Fig. 2B). Comparable
results were obtained in ER-negative U2-OS osteosarcoma cells
(Fig. 2C, D), indicating that the effect of cyclin D1 is not
cell type-specific. These results indicate that cyclin D1 can
5   mediate activation of ER mutants that are unable to interact
with SRCs efficiently.

To ask whether SRCs are involved in the cyclin D1-mediated
activation of the mutant ERs, a dominant negative mutant of
SRC-1 (SRC1-DN, encoding amino acids 1245-1441 of SRC-1) was
10   used. This mutant harbors a LXXLL motif, which mediates binding
to the leucine-rich coactivator binding site in ER (Heery et
al., 1997; Le Douarin et al., 1995) but lacks a transactivation
domain (Jenster et al., 1997; Spencer et al., 1997). As shown
in previous studies (Onate et al., 1995), this construct served
15   as a dominant inhibitor for endogenous SRC-1 function on wild
type ER (Fig 3A). As expected, SRC1-DN inhibited the ability of
SRC-1 and of the closely related coactivator TIF2 on ER
transactivation (Fig. 3B), whereas it was inactive on the
non-related E2F-1 transcription factor (data not shown) .
20   Importantly, SRC1-DN markedly repressed the cyclin D1-induced
activation of the ER AF-2 mutants (Fig. 3C, D). These data
suggest that the cyclin D1-mediated activation of these mutant
ERs somehow requires SRC activity.

Direct binding of cyclin D1 to steroid receptor coactivators.
25   Since the leucine-rich motifs of nuclear receptors have been
shown to recruit SRC family coactivators (Danielian et al.,
1992; Heery et al., 1997), we tested whether cyclin D1 is also
able to interact with SRCs through its leucine-rich motif.
COS-7 cells were cotransfected with HA-tagged constructs
30   encoding the nuclear receptor coactivators SRC-1, AIB-1 or p300
(Anzick et al., 1997; Chakravarti et al., 1996; Hanstein et
al., 1996; Kamei et al., 1996; Onate et al., 1995) together
with control plasmid, plasmids directing the synthesis of wild
type cyclin D1 or D1 L254/255A mutant (D1-LALA). 12CA5 HA
35   antibodies were used for immunoprecipitation of whole cell
extracts prepared from these cells and co-immunoprecipitation
of cyclin D1 (mutants) was examined by Western blot analysis

using anti-cyclin D1 antibody. The results showed that wild-
type cyclin D1, but not the leucine-to-alanine mutant D1-LALA,
co-immunoprecipitates with SRC-1 and AIB-1, whereas binding of
both wild type cyclin D1 and D1 LALA mutant to p300 was hardly

5   detectable. Precipitation with the anti-cyclin D1 antibody
from the total lysate of the cells transfected with cyclin D1
and cyclin D1-LALA was similar. Binding of cyclin D1 and
cyclin D1 L254/255A mutant to ER was then investigated. COS-7
cells were transfected with ER expression vector, cyclin D1

10 (mutant) and/or control plasmids. Monoclonal ER antibodies were
used for immunoprecipitation of ER of whole cell extracts
prepared from these cells and co-immunoprecipitation of cyclin
D1 (mutant) was examined by Western blot analysis using
monoclonal cyclin D1 antibodies. The results show that co-

15 immmunoprecipitation was similar for the (ER and cyclin D1) and
(ER and cyclin D1-LALA) samples, showing that the mutant was
expressed equally and was unaffected in its ability to bind ER.
The activity of cyclin D1 and D1 L254/255A mutant in
phosphorylation of pRb in $Rb^{-/-}$ 3T3 cells was also investigated.

20 Cells were transfected with pRb expression vector, cyclin D1
(mutant) and/or control plasmids and were maintained in low
serum conditions. After 40 hours, cells were lysed and proteins
were separated by low percentage polyacrylamide gel
electrophoresis. Differentially phosphorylated species of pRb

25 were detected by Western blotting using the polyclonal pRb
antibody (C15, Santa Cruz). Strong bands were seen in the
Rb/cyclin D1 and Rb/cyclin D1-LALA bands but not in the control
Rb band showing that the mutant was unaffected in its ability
to phosphorylate pRb in cells that lack cyclin D1-associated

30 kinase activity.

In summary, these three experiments show that the cyclin D1
mutant L254/255A, which failed to activate ER (Fig. 1B), did
not interact with SRC family proteins, though this mutant was
expressed equally and was unaffected in its ability to bind ER

35 and to phosphorylate pRb in cells that lack cyclin
D1-associated kinase activity. These data suggest that the
AF-2-like leucine-rich motif of cyclin D1 mediates binding to
SRC-family proteins.

To test whether the interaction between cyclin D1 and SRC-1 is
direct, we used bacterially expressed GST-SRC1 and
E.coli-produced 6X histidine-tagged cyclin D1 in an in vitro
protein binding assay. As described below, His-cyclin D1

5   strongly binds to GST-SRC1 protein, but not to GST alone,
indicating that this association is specific and direct.

To ask which domain or motif of SRC-1 is involved in cyclin D1
binding, we generated a series a GST-SRC1 deletion mutants.
The series of GST fusion protein containing SRC-1 (GST-SRC1:

10   361-1441; GST-SRC1 361-782; GST-SRC1 361-568) or GST-p300
(1-595) were tested for direct binding to His-tagged cyclin D1
(His-D1); the GST-SRC1 derivatives are shown in Figure 4A. The
conserved LXXLL motif are boxed and the amino acid boundaries
are demonstrated. In the in vitro binding assay, the series of

15   GST-containing proteins were incubated with bacterially
expressed His-tagged cyclin D1 and immobilized on
glutathione-agarose. Cyclin D1 binding was detected by Western
blot analysis using anti-cyclin D1 antibody. Binding was not
detected to GST alone. Binding was detected to the GST-SRC1

20   361-1441, GST-SRC1 361-782 but not to GST-SRC1 361-568 or
GST-p300 (1-595) proteins, showing that amino acids 568 to 782
of SRC-1 are required for cyclin D1 binding. Interestingly,
this region of SRC-1 harbors three LXXLL motifs, which are
involved in binding to nuclear receptors (Heery et al., 1997;

25   Le Douarin et al., 1995; Torchia et al., 1997).

To ask whether the LXXLL motifs of SRC-1 are involved in cyclin
D1 binding, a peptide competition experiment was performed.
Peptides (0.3 μg and 3 μg) derived from the four LXXLL motifs
of SRC1 were used in a GST pull down assay using GST-SRC1

30   (361-1441) and His-tagged cyclin D1 as described above. The
sequence and the position of the peptides in SRC-1 are shown in
Figure 4B. The results of the assay showed that LXXLL
peptides, but not a LXXAA mutant peptide, interfered with
binding of cyclin D1 to SRC-1. The reduction in binding was

35   greater with 3 μg than with 0.3 μg of each of the LXXLL
peptides with no reduction shown with the LXXAA mutant peptide.
Of the four SRC-1 LXXLL peptides tested, the P3 peptide, which

corresponds to the third of the three centrally located LXXLL
motifs, was the best competitor. In another experiment, the
ability of peptides to compete with cyclin D1 for binding to
GST-SRC1 was investigated. Increasing amounts (100,200 and 500

5   nM) of AASKHKQLSELLRSG (LXXLL) and AASKHKQLSEAARSG (LXXAA)
peptides were used in a GST pull down assay using GST-SRC1
(361-1441) and His-tagged cyclin D1. Binding was reduced with
increasing concentration of LXXLL peptides but not with
increasing concentration of LXXAA peptides. These results

10   indicate that SRC-1 uses the LXXLL motifs not only to interact
with nuclear receptors, but also to bind cyclin D1. Since these
LXXLL motifs interact with the leucine-rich motifs on nuclear
receptors (Heery et al., 1997; Torchia et al., 1997), these
data are in good agreement with our experiments described above

15   which suggested a major role for the leucine-rich AF-2-like
motif of cyclin D1 in SRC binding. Since both the in vitro and
the in vivo association experiments described above were
performed in the absence of 17β-estradiol, these data also
indicate that the cyclin D1/SRC-1 interaction, in contrast to

20   the ER/SRC-1 interaction, is hormone-independent.

Cyclin D1 act as a physical bridge between ER and SRC-1.

Cyclin D1 activates the estrogen receptor (ER) in a
ligand-independent fashion through direct binding to ER (Neuman
et al., 1997; Zwijsen et al., 1997). The present study

25   demonstrates that cyclin D1, besides ER, also interacts
directly with SRC-1 in vivo and in vitro. These data suggest a
model in which cyclin D1 can act as a bridging factor between
ER and SRC-1, allowing ligand-independent recruitment of
coactivators to ER in the presence of cyclin D1. To test this

30   model directly, we used bacterially expressed 6X
histidine-tagged cyclin D1, E.coli-produced GST-SRC1 and
baculovirus-produced ER in an in vitro GST pull down assay.
Protein binding to GST-SRC1 was identified by Western blotting
analysis using monoclonal antibodies directed against cyclin D1

35   and ER. GST protein served as a control for binding
specificity. In agreement with several earlier studies, we
found that ER binds to GST-SRC1 in a ligand-dependent manner in
vitro (Cavailles et al., 1994) (Fig. 5A). Significantly, cyclin

D1 could also recruit ER to GST-SRC1 in the absence of ligand
(Fig. 5A). These data indicate that cyclin D1 can cause ER
activation by acting as a ligand-independent adapter molecule
between ER and its coactivator SRC-1.

5   To test whether a ternary complex can also be formed when ER is
bound to its cognate DNA binding site (the ERE), we performed a
similar ternary complex assay with purified proteins as
described above, with the modification that the ternary complex
was pulled down with ERE oligonucleotides coupled to
10   paramagnetic beads. Fig 5B shows that ER binds to ERE in the
presence of ligand (lanes 3-6), but also in the absence of
ligand when cyclin D1 is present (lanes 8 and 10). Thus, cyclin
D1 allows ligand-independent DNA binding of ER to its ERE. More
importantly, this assay clearly shows that a ternary complex
15   consisting of ER, cyclin D1 and ER can be formed on DNA both in
the presence and in the absence of 17β-estradiol (lanes 6 and
10). These data suggest that cyclin D1 is a bridging factor
between ER and SRC-1 also when ER is bound to DNA.

The role of cyclin D1 in ER activation in breast cancer.
20   To get more insight in the role of cyclin D1 in ER
transactivation in breast cancers with elevated levels of
cyclin D1, we have tested the effect of the mutant cyclin D1
L254/255A on ER activation in two different breast cancer cell
lines. This cyclin D1 mutant does bind to ER, but fails both to
25   interact with SRC-1 as shown above and consequently fails to
activate ER (Fig. 1B). Therefore, this mutant can act as a
dominant negative in cyclin D1-mediated ER transactivation as
it binds to ER and fails to recruit coactivators. We tested
the effect of this cyclin D1 mutant on ER activation in two
30   breast cancer cell lines, T47D and MCF-7, which contain
endogenous wild type ER, but differ in their cyclin D1 protein
levels (Fig. 6).In T47D, which contain relative low levels of
cyclin D1, co-expression of this cyclin D1 mutant resulted in a
slight inhibition of ER activity. In contrast, in MCF-7, which
35   contain relative high levels of cyclin D1 (Fig.6), the cyclin
D1 mutant inhibited up to 50% of ER activity. Thus, this
dominant negative cyclin D1 mutant preferentially interferes

with ER activation in breast cancer cells that have high levels
of cyclin D1 protein. These data strongly support the notion
that elevated cyclin D1 protein levels in breast cancer
contribute significantly to ER activation.

5   In parallel experiments, we have used the above dominant
    negative mutant of cyclin D1 to show that activation of the
    estrogen receptor complex by D1 stimulates the cell cycle.
    Cell cycle profiles were analysed using fluuorescence activated
    cell sorting (FACS)(Allen, 1990; Baldetorp et al, 1998).

10 Transient transfection of MCF-7 breast cancer cells with the
    L254/255A mutant of cyclin D1 causes an arrest in the cell
    cycle at the G1/S checkpoint (Table 2), thus confirming the
    role of the estrogen receptor-cyclin D1 interaction in cell
    proliferation.

15

Table 2			
Phase of cell cycle	G0/G1	S	G2/M
No cyclin D1	73.4%	18.8%	7.8%
Cyclin D1	76.5%	16%	7.6%
Cyclin D1-LALA	81.9%	10.2%	7.9%

20 In summary, the invention indicates that cyclin D1 can act as a
    bridging factor between ER and steroid receptor coactivators
    (SRCs) which allows the formation of a transcriptionally active
    ternary complex in the absence of ligand (Fig. 7). It is
    generally thought that coactivator recruitment by nuclear
25 receptors results from a ligand-induced conformational change
    in the AF-2 domain of the receptor (Brzozowski et al., 1997;
    Renaud et al., 1995). Our present data for the first time show
    an alternative route of coactivator recruitment to ER that can
    take place in the absence of ligand. As such, these data reveal
30 a novel mechanism of ER activation and establish a direct role
    for cyclin D1 in regulation of transcription.

Our work has led to the identification of a novel functional
domain in the carboxyl terminus of cyclin D1 that mediates
direct binding to steroid receptor coactivators like SRC-1 and
AIB-1. This leucine-rich motif of cyclin D1 is very similar in
5   character to the ligand-regulated steroid receptor coactivator
binding motif that is present in helix 12 of ER and in many
other nuclear receptors. Several lines of experimental evidence
indicate that the leucine-rich motif of cyclin D1 is required
for coactivator recruitment to ER and subsequent activation of
10 ER. First, cyclin D1 interacts directly with SRC-1 both in vivo
and in vitro and introduction of two point mutations in this
motif of cyclin D1 abolishes SRC-1 interaction and prevents ER
activation by cyclin D1. Second, cyclin D2 and D3 have only a
partial conservation of the leucine-rich motif and are hardly
15 active in ER activation (Neuman et al., 1997; Zwijsen et al.,
1997). Third, a dominant negative mutant of SRC-1 prevents
cyclin D1 activation of ER, indicating that SRCs are required
for cyclin D1-mediated activation of ER (Fig. 3). Fourth, in in
vitro binding studies cyclin D1 could recruit ER to SRC-1 in
20 the absence of ligand (Fig. 5). Together these data suggest a
model in which cyclin D1 can recruit SRCs to ER which results
in a transcriptionally productive interaction between ER and
its coactivators.

The functional similarity between the leucine-rich motifs of
25 cyclin D1 and ER is also supported by structural analysis. Even
though a crystal structure of cyclin D1 is not available at
present, the crystal structures of cyclins A and H have been
solved (Andersen et al., 1997; Jeffrey et al., 1995). Alignment
of the sequence of cyclin D1 with cyclin A and comparison with
30 the structures of cyclins A and H indicates that the
leucine-rich motif in the carboxyl terminus of cyclin D1 aligns
at the C-terminus of helix 5' of cyclins A and H. Importantly,
the PHD program indicates that this part of cyclin D1 has a
more than 90% probability to be a-helical and is markedly
35 amphipathic (Rost and Sander, 1993). Since the leucine-rich
coactivator binding motif of ER is also an amphipathic helix,
it is well-possible that the leucine-rich motifs of cyclin D1
and ER are capable of making similar protein interactions with

SRCs.

Consistent with the notion that cyclin D1 and ER have a similar
coactivator interaction surface, we found that binding of
cyclin D1 to SRC-1 also requires the highly conserved LXXLL

5   motifs in SRC-1. These motifs were recently shown to mediate
binding to the leucine-rich coactivator binding site in the
amphipathic helix 12 of ER (Heery et al., 1997; Le Douarin et
al., 1995; Torchia et al., 1997). Significantly, depending on
the pattern of splicing, SRC-1 has three or four LXXLL motifs,

10 three of which are in close proximity (Kalkhoven et al., 1998).
In principle, this could allow simultaneous interaction of
SRC-1 with the leucine-rich motifs of both ER and cyclin D1.
Consistent with this, we observed that a peptide that spans the
third LXXLL motif of SRC-1 competed most efficiently the

15 binding between cyclin D1 and SRC-1, whereas previous studies
have indicated that the second LXXLL motif of SRC-1 is the
preferred site of interaction for ER (Heery et al., 1997;
Kalkhoven et al., 1998). Based on these observations we propose
that in the absence of ligand, expression of cyclin D1 provides

20 a single interaction site for coactivators on the cyclin D1/ER
complex as both binding of cyclin D1 to ER and binding of
cyclin D1 to SRC-1 is ligand-independent. This provides a
rationale for the ligand-independent activation of ER in the
presence of high levels of cyclin D1 (Zwijsen et al., 1997).

25 After ligand binding of ER, the leucine-rich domain in AF-2 is
exposed which constitutes a second binding site for SRCs. The
presence of two SRC-1 binding sites on the liganded cyclin
D1/ER complex provides a rationale for the observed synergism
between estradiol and cyclin D1 in ER activation (Zwijsen et

30 al., 1997, Fig. 7).

The model represented in Fig. 7 does not take into account that
binding of cyclin D1 to ER also allows ligand-independent DNA
binding by ER in vitro and in vivo (Fig. 5B and Zwijsen et al.,
1997). Thus, cyclin D1 can not only stimulate coactivator

35 recruitment to ER but also act to enhance DNA binding of ER.
Thus, the synergistic action between cyclin D1 and ligand in ER
activation may also be due, in part, to synergistic induction

of ER DNA binding (Fig. 2 and Zwijsen et al., 1997).

The present study showed that cyclin D1 can bind to SRC-1 and
AIB-1, but not to p300. Thus, cyclin D1 can discriminate
between the different coactivator families. Apparently, a LXXLL
5   motif (present in both SRCs and p300) is required for cyclin D1
binding, but flanking regions contribute to binding specificity
(see also Fig .4B). The finding that cyclin D1 interacts with
at least two members of the SRC family, SRC-1 and AIB-1, would
allow in principle for promiscuous activation of steroid
10   receptors by cyclin D1. However, cyclin D1 does not activate
the progesterone receptor, nor a number of other steroid
hormone receptors (Zwijsen et al., 1997) (R.M.L.Z. and R.B,
unpublished data). It is likely that the ability of cyclin D1
to interact with ER directly contributes to the specificity of
15   nuclear receptor activation by cyclin D1.

Cyclin D1 is an important regulator of growth and
differentiation of breast epithelium (Fantl et al., 1995;
Musgrove et al., 1994; Sicinski et al., 1995; van Diest et al.,
1997; Wang et al., 1994; Zwijsen et al., 1996). Significantly,
20   both the genes encoding cyclin D1 and the steroid receptor
coactivator AIB-1 are frequently amplified or overexpressed in
breast cancer (Anzick et al., 1997; Buckley et al., 1993;
Gillett et al., 1994; Schuuring et al., 1992; van Diest et al.,
1997). Since the present study indicates that both cyclin D1
25   and SRCs are components of a multimeric complex involved in
ER-mediated transcription, it is conceivable that
over-expression of limiting factors in this complex results in
deregulation of ER-mediated growth. In agreement with this, we
found that a mutant of cyclin D1 that can bind to ER but fails
30   to recruit coactivators acted as a dominant negative mutant for
ER activation primarily in breast cancer cells with elevated
levels of cyclin D1. Thus, cyclin D1 is likely to contribute
significantly to ER activation in breast cancers in which the
protein is over-expressed. An important question that we wish
35   to address next is how much of the oncogenic activity of cyclin
D1 in breast cancer is mediated through the "classical" cdk4
route and how much through ER activation. The availability of

specific mutants of cyclin D1 in which these activities can be
separated should allow us to assess the contribution of each of
these two activities of cyclin D1 to mammary carcinogenesis
separately.

5   **Experimental Procedures**

Cell culture and transient transfection assays.

COS-7 cells and U2-OS cells were maintained in DMEM
supplemented with 10% fetal bovine serum. Twenty-four hours
before transfection, cells were maintained in DMEM without
10   phenol red containing 5% charcoal-treated FBS. Cells were
transfected with 3 μg ERE-TATA-luciferase expression vector,
500 ng β-galactosidase expression vector (internal control),
200 ng ER expression plasmid and 2.5 μg cyclin D1, coactivators
and/or empty vectors as indicated. After sixteen hours, cells
15   were rinsed in PBS and re-fed with fresh medium and ligand (10
nM 17β-estradiol) or vehicle was added. One day later, cells
were harvested and assayed for luciferase and β-galactosidase
activities. β-galactosidase activity was used to correct for
differences in transfection efficiency.

20   Immunoprecipitation and Western blotting.
Cells were lysed in ELB containing 250 mM NaCl, 0.1% NP-40, 50
mM HEPES pH 7.0, 5 mM EDTA and protease inhibitors. The cell
lysate was pre-cleared three times with 5 μl of normal mouse
serum coupled to protein A-Sepharose beads. For
25   immunoprecipitations, the supernatant was incubated with 100 μl
of 12CA5 hybridoma supernatant or 10 μl monoclonal antibody to
the estrogen receptor (TE111.5D11, Neomarkers), which was
coupled to protein A sepharose beads at 4°C. After 1 hour,
beads were washed in ELB buffer and boiled in Laemmli-buffer.
30   Samples were separated on a 10% SDS/polyacrylamide gel and
transferred to nitrocellulose. After blocking with PBS
containing 5% milk and 0.1% Tween-20, proteins were detected
with monoclonal antibodies directed against cyclin D1 (DCS-6,
Neomarkers) and peroxidase-conjugated goat anti-mouse IgG. The
35   blots were washed in PBS containing 0.1% Tween-20 and developed
by enhanced chemiluminescence (ECL) reactions (Amersham).

DNA binding assay.

For the DNA binding assay we used DNA affinity beads coated
with streptavidin (Dynal A/S) and (5 -biotin-labeled) DNA
oligonucleotides containing a binding sequence for ER as
5   described before (Zwijsen et al., 1997). The complementary DNA
strands were annealed in TE buffer containing 100 mM KCl at 75
ûC for 10 min. followed by cooling to room temperature over a
period of 2 h. Dynabeads were mixed with biotinylated
oligonucleotides in TE buffer containing 1 M NaCl for 15 min.,
10   washed and incubated with cell extract in 8 mM Tris-phosphate
pH 7.4, 0.12 KCl, 8% glycerol, 4 mM DTT and 0.5% CHAPS for 1 h
at 4°C. Subsequently, beads were washed in 20 mM Hepes pH 7.7,
50 mM KCl, 20% glycerol and 0.1% NP-40. The beads were boiled
in Laemlli buffer and the proteins were separated on 10% PAGE
15   and identified by Western blotting.

GST pull down and peptide competition assay.

GST protein, GST-SRC1 fusion protein and His-tagged cyclin D1
protein were purified as described previously (Zwijsen et al.,
1997). Binding between 500 ng GST-SRC1 and 100 ng His-D1 was
20   performed in binding buffer (50 mM NaCl, 50 mM HEPES-KOH pH
7.6, 0.1 mM 0.1% (w/v) NP40, 0.1 mM PMSF and 0.5%
charcoal-stripped serum) bound to glutathione-sepharose for 1
hour at 4°C. The beads were washed three times and bound
proteins were eluted by boiling for 10 minutes in sample buffer
25   and separated on 10% SDS-PAGE. The binding of His-cyclin D1 to
GST-SRC1 was detected by Western blot analysis using monoclonal
antibodies directed against cyclin D1 (DCS-6, Neomarkers). For
testing a ternary complex, a baculovirus-produced ER (750 ng,
Pan Vera) was added to GST-SRC1 (500 ng) and His-D1 (100 ng)
30   in the presence or absence of 1 μM 17β-estradiol using similar
conditions as described above. In Western blot analysis
monoclonal antibodies directed against cyclin D1 (DCS-6,
Neomarkers) and ER (LH2, Novocastra) were used.

For the peptide inhibition assay using the peptides shown in
35   Fig 4(B), 150 ng GST/GST-SRC1 and 50 ng His-tagged cyclin D1
were used. Peptides were pre-incubated with target protein for
40 minutes at room temperature, prior to addition of the bait.

A mixture of GST-fusion peptides, His-tagged cyclin D1 and
peptides was incubated for an additional 20 min. at room
temperature. The amount of peptides added in competition
studies were 0.3 μg and 3 μg. For the peptide inhibition assay
using the peptides AASKHKQLSELLRSG and AASKHKQLSEAARSG derived
from p300 amino acid 74-88, the peptides were pre-incubated
with target protein for 20 minutes at room temperature, prior
to addition of the bait. The peptides added in competition
studies were 100 nM, 200 nM and 500 nM.

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reference.

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CLAIMS

1.   An assay for a modulator of estrogen responsive tumour
cells which comprises:
a)   bringing into contact a cyclin D1, an SRC and a putative
      imodulator compound under conditions where the cyclin D1
      and the SRC, in the absence of inhibitor, are capable of
      forming a complex; and
b)   measuring the degree of inhibition of complex formation
      caused by said modulator compound.

2.   An assay for a modulator of estrogen responsive tumour
cells which comprises:
a)   bringing into contact a cyclin D1, an SRC, an estrogen
      receptor and a putative inhibitor compound under
      conditions where the cyclin, the SRC and the estrogen
      receptor, in the absence of inhibitor, are capable of
      forming a complex which is capable of binding to an
      estrogen response element;
b)   providing an estrogen response element to which the
      complex is capable of binding and transcriptionally
      activating; and
c)   measuring the degree of inhibition of binding or
      transcriptional activation caused by said inhibitor
      compound.

3.   An assay according to claim 1 which is in the form of a
two-hybrid assay.

4.   An assay according to any one of the preceding claims
which is performed in vivo in an estrogen responsive cell line.

5.   An assay according to any one of the preceding claims in
which an estrogen is also present.

6.   An peptide of formula:

$$Z^1-LXXLL-Z^2$$

where Z^1 is an N-terminal of a sequence of from 1 to 8 amino

acids, and Z² is a C-terminal or sequence of from 1 to 8 amino
acids.

7.   A peptide according to claim 6 wherein Z¹ and/or Z²
represent a sequence found immediately N-terminal and/or C-
terminal respectively to a naturally occuring LXXLL motif in an
SRC or other nuclear receptor interacting protein.

8.   A peptide according to claim 6 wherein Z¹ comprises from 3
to 7 amino acids and contains at least one residue selected
from the group of K and H.

9.   A peptide of a formula selected from the group:
A A S K H K Q L S E L L R S G (SEQ ID NO:1)
        S H K L V Q L L T T T A E Q (SEQ ID NO:2)
    E R H K I L H R L L Q E G S (SEQ ID NO:3)
      K D H Q L L R L L D K D E (SEQ ID NO:4); and
P Q A Q Q K S L Q Q L L T   (SEQ ID NO:5)

10.   A peptide whose sequence comprises a peptide as defined in
any one of claims 6 to 9 joined to a membrane translocation
sequence.

11.   A composition comprising a peptide according to any one of
claims 6 to 9 together with a diluent or carrier.

1/10

FIG. 1(A)
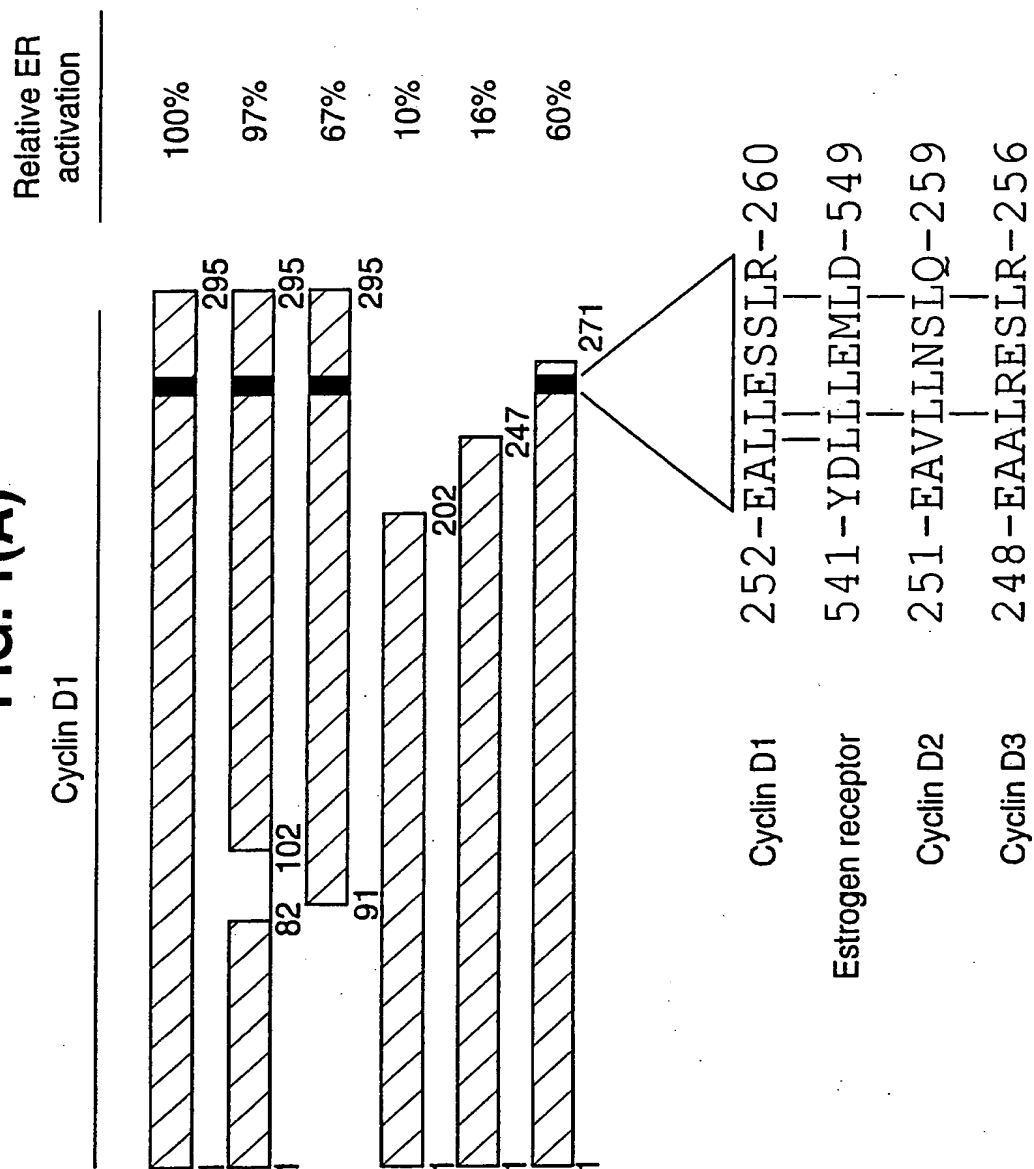
FIG. 1(B)
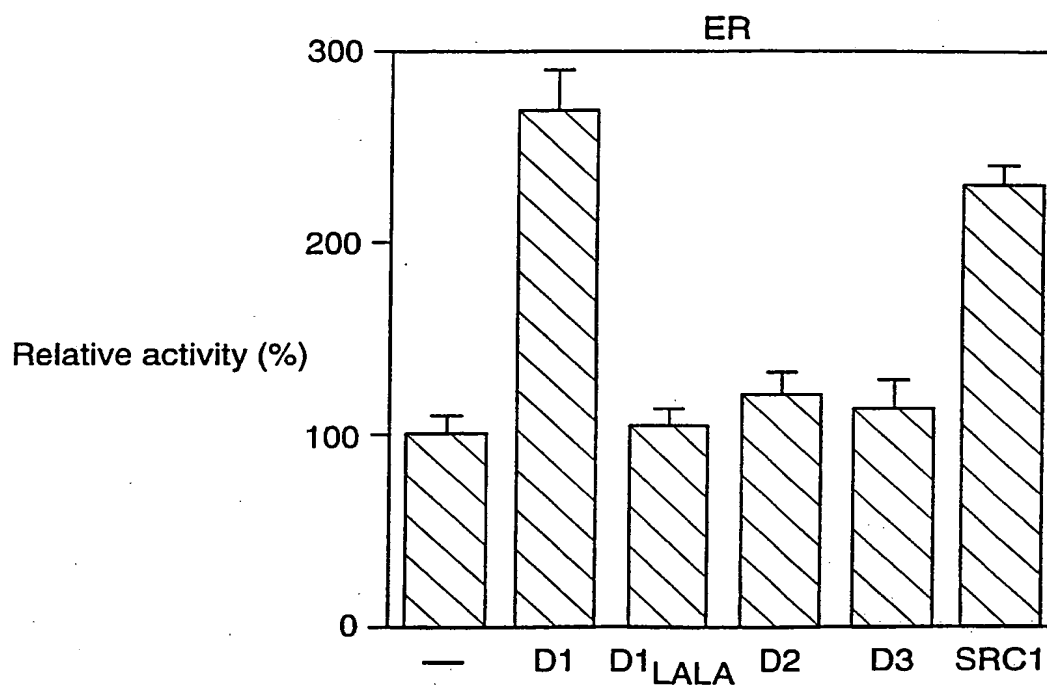
FIG. 2(A)
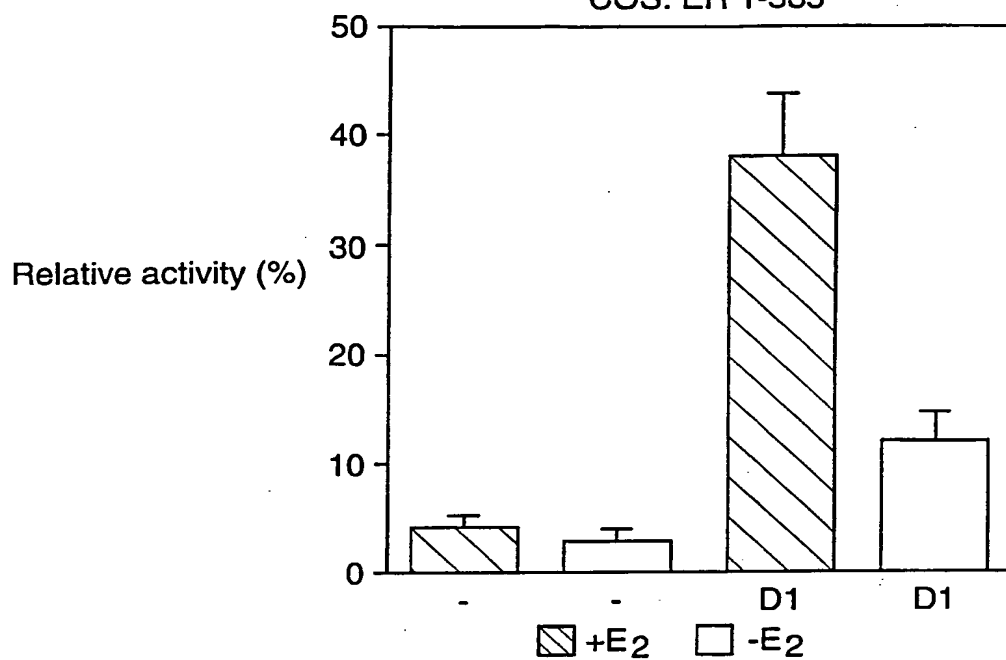
COS: ER 1-535

FIG. 2(B)

COS: ER L543/544A
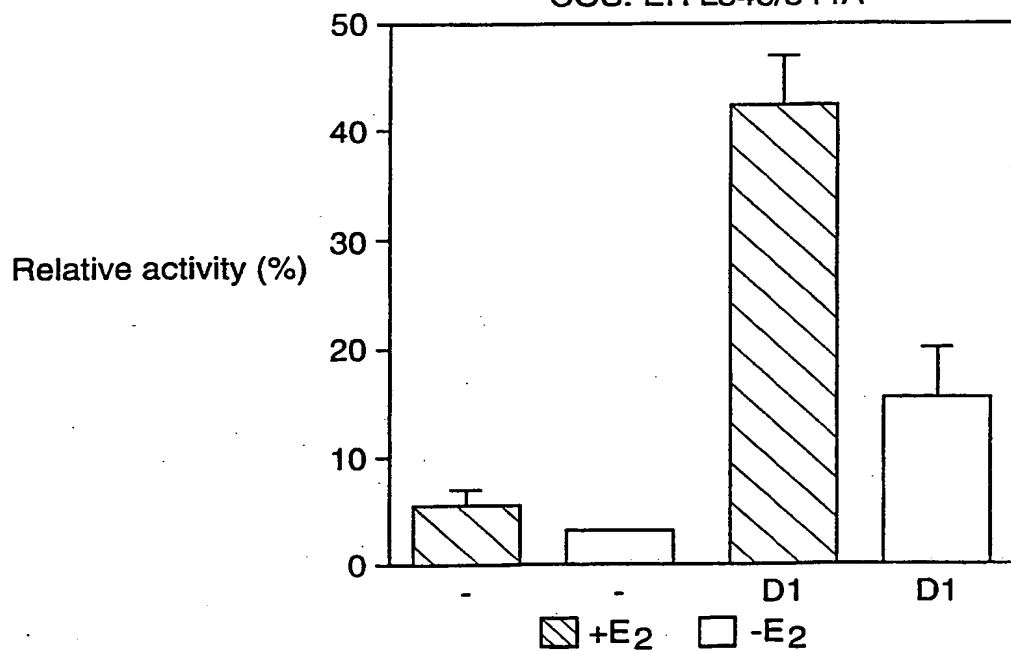
FIG. 2(C)

U2OS: ER 1-535

FIG. 2(D)

U20S: ER L543/544A

FIG. 3(A)

FIG. 3(B)

FIG. 3(C)

ER 1-535

Relative activity

—   SRC1 DN   cyclin D1

SRC1 DN

FIG. 3(D)

ER L543/544A

Relative activity

—   SRC1 DN   cyclin D1

SRC1 DN

SUBSTITUTE SHEET (rule 26)

FIG. 4(A)

FIG. 4(B)

PEPTIDE	SEQUENCE		POSITION IN SRC-1
P1	SHK	L VQ LL	TTTAEQ	630-643
P2	ERHKI	L HR LL	QEGS	685-698
P3	KDHQL	L RY LL	DKDE	744-757
P4	PQAQQKS	L QQ LL	T	1427-1440
P4M	PQAQQKS	L QQ AA	T	—

FIG. 5(A)

FIG. 5(B)

FIG. 6

FIG. 7

(51) International Patent Classification 6 : G01N 33/50, 33/74, C07K 4/12, 7/08	A3	(11) International Publication Number: WO 99/41608
		(43) International Publication Date: 19 August 1999 (19.08.99)

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(74) Agents: BRASNETT, Adrian, H. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).

(54) Title: INTERACTION BETWEEN CYCLIN D1 AND STEROID RECEPTOR CO-ACTIVATORS

(57) Abstract

    The present invention relates to the finding that cyclin D1 interacts in a ligand–independent fashion with coactivators of the SRC–1 family. The direct interaction of cyclin D1 enhances estrogen receptor (ER) mediated transcription and provides a novel target for the development of assays for substances which modulate the cell cycle. The invention provides assay methods for the prevention of growth of tumours, for assays for compounds useful in the prevention of tumours and compounds obtainable by such assays.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   G01N33/50   G01N33/74   C07K4/12   C07K7/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 40378 A (VER NL KANKER INST ;BERNARDS RENE (NL); MICHALIDES ROB J A M (NL);) 30 October 1997 (1997-10-30) cited in the application claim 1	1-5
A	HEERY, DAVID M. ET AL.: "A signature motif in transcriptional co-activators mediates binding to nuclear receptors" NATURE, vol. 387, 12 June 1997 (1997-06-12), pages 733-736, XP002107158 cited in the application the whole document	1-5

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 June 1999	3 0. 09. 1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gundlach, B

Form PCT/ISA/210 (second sheet) (July 1992)

Int .tional Application No

PCT/GB 99/00440

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	ZWIJSEN R M L ET AL: "CDK-INDEPENDENT ACTIVATION OF ESTROGEN RECEPTOR BY CYCLIN D1" CELL, vol. 88, no. 3, 7 February 1997 (1997-02-07), pages 405-415, XP002038473 cited in the application the whole document -----	1-5

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INTERNATIONAL SEARCH REPORT	.rnational application No.
	PCT/GB 99/00440

Box I   Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

:see further information

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

  1-5

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

1. Claims: 1-5

   Identification of substances that inhibit the complex
   formation of protein SRC and cyclin D1 in estrogen
   responsive tumour cells

2. Claims: 6-11

   Peptides with the formula Z1-LXXLL-Z2 (Z1 and Z2 = 1-8 amino
   acids)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9740378	A	30-10-1997	AU	2698197 A	12-11-1997
			CA	2249396 A	30-10-1997
			EP	0898708 A	03-03-1999